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(71) Applicant *(for all designated States except US)*: PURDUE RESEARCH FOUNDATION [US/US]; 1021 Hovde Hall, Room 307, West Lafayette, IN 47907-1021 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: THOMPSON, David, H. [US/US]; 230 Spring Valley Lane, West Lafayette, IN 47906 (US). LOW, Philip S. [US/US]; 5850 Farm Ridge Road, West Lafayette, IN 47906 (US). RUI, Yuanjin [CN/US]; Apartment 129, 8148 Genesee Avenue, San Diego, CA 92122 (US). WANG, Susan [CN/US]; 3366 Peppermill Drive, West Lafayette, IN 47906 (US).

(74) Agents: LAMMERT, Steven, R. et al.; Barnes & Thornburg, 1313 Merchants Bank Building, 11 South Meridian Street, Indianapolis, IN 46204 (US).

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(57) Abstract

An improved liposome and method for delivering an exogenous molecule to the cytoplasm of a cell is described. The liposomal membrane comprises triggerable lipids and lipids complexed to a ligand, wherein the ligand is capable of interacting with cellular membrane to enhance the uptake of the ligand and attached liposome.

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LIPOSOMAL DELIVERY SYSTEM

Background of the Invention

The present invention is directed to liposomes, and more particularly, a liposomal delivery system and method for transporting materials such as drugs, nucleic acids, and proteins to a targeted population of cells. The liposomes of the present invention comprise modified lipids that enhance the delivery of exogenous molecules encapsulated therein to the cytoplasm of cells.

Liposomes are microscopic lipid bilayer vesicles that enclose a cavity. The liposomal vesicles can contain a single phospholipid bilayer (unilamellar vesicle) or multiple phospholipid bilayers (multilamellar vesicle). Liposome technology has been applied to the formulation and delivery of pharmaceutics, diagnostic imaging, clinical analysis, cosmetics, food processing and cellular transfection. For example, U.S. Pat. No. 3,993,754 discloses an improved chemotherapy method for treating malignant tumors in which an anti-tumor drug is encapsulated within liposomes and the liposomes are injected into an animal. Furthermore, encapsulation of pharmaceuticals in liposomes can reduce drug side effects, improve pharmacokinetics of delivery to a target site, and improve the therapeutic index of a drug.

Previous studies with phospholipid-based liposomes have established that they possess low acute toxicity, are readily biodegradable, and are deposited primarily in the liver, spleen, reticuloendothelial system, and in tumor neovasculature. Blood circulation times, tissue distribution, and nonspecific cellular responses can be manipulated experimentally. Recently reported formulations incorporating minor proportions (0.5-10 mol%) of gangliosides or poly(ethylene glycol) - (PEG)

derivatized lipids (i.e., sterically stabilized liposomes bearing MW 1000-5000 PEG chains on the liposomal membrane surface) have greatly extended blood circulation times and reportedly improved the passive targeting of liposomes to tumor sites.

The delivery of administered liposomal carriers to a cell can be enhanced by attaching or adsorbing various ligands to the exterior surface of the liposomal vesicle (For an overview see Martin, F.J., et al. Liposomes a Practical Approach (New, R.R.C., Ed) pages 163-182, IRL Pres, Oxford (1990). The ligand can be attached (through covalent, hydrogen or ionic bonds) to the phospholipids forming the liposome either by direct linkage or by connection through intermediary linkers, spacer arms, bridging molecules. Alternatively, the ligand can be anchored into the liposome bilayer through hydrophobic interactions.

Generally, a specified ligand is chemically conjugated by covalent, ionic or hydrogen bonding to the liposomal surface of a liposome by forming a conjugate having a moiety (the ligand portion) that is still recognized in the conjugate by a target receptor. Using this technique the phototoxic compound psoralen has been conjugated to insulin and internalized by the insulin receptor endocytotic pathway (Gasparro, Biochem. Biophys. Res. Comm. 141(2), pp. 502-509, Dec. 15, 1986); the hepatocyte specific receptor for galactose terminal asialoglycoproteins has been utilized for the hepatocyte-specific transmembrane delivery of asialoorosomucoid-poly-L-lysine non-covalently complexed to a DNA plasmid (Wu, G.Y., J. Biol. Chem., 262(10), pp. 4429-4432, 1987); the cell receptor for epidermal growth factor (EGF) has been utilized to deliver polynucleotides covalently linked to EGF to the cell interior (Myers, European Patent Application 86810614.7, published June 6,

1988); the intestinally situated cellular receptor for the organometallic vitamin B_{12}-intrinsic factor complex has been used to mediate delivery to the circulatory system of a vertebrate host a drug, hormone, bioactive peptide or immunogen complexed with vitamin B_{12} and delivered to the intestine through oral administration (Russell-Jones et al., European patent Application 86307849.9, published April 29, 1987); the mannose-6-phosphate receptor has been used to deliver low density lipoproteins to cells (Murray, G. J. and Neville, D.M., Jr., J.Bio.Chem, Vol. 255 (24), pp. 1194-11948, 1980); the cholera toxin binding subunit receptor has been used to deliver insulin to cells lacking insulin receptors (Roth and Maddox, J.Cell.Phys. Vol. 115, p. 151, 1983); and the human chorionic gonadotropin receptor has been employed to deliver a ricin a-chain coupled to HCG to cells with the appropriate HCG receptor in order to kill the cells (Oeltmann and Heath, J.Biol.Chem, vol. 254, p. 1028 (1979)).

Vitamins such as thiamin, folate, biotin, and riboflavin have also been used to enhance the uptake of exogenous molecules (US Patent No. 5,108,921 and 5,416,016).

Liposome Preparation

General methods of making liposomes are known. See for example U.S. Pat. No. 4,882,165, and Deamer and User, "Liposome Preparation: Methods and Mechanisms," in *Liposomes*, Marcel Dekkev, Inc., New York (1983), both of which are incorporated herein by reference. Liposomes may be produced by a wide variety of methods. Multilamellar vesicles (MLV) are formed by simple hydration of dry lipid powders. The particles formed are typically quite large (>10μm) and are often oligolamellar (i.e., possessing more than one bilayer membrane). This method is most commonly used to produce giant, unilamellar liposomes for micropipet

measurements to determine the mechanical properties of bilayer membranes. Ultrasonication with probe type sonicators or processing through a French press produces small, unilamellar vesicles (SUV) with average diameters in the 25-50 range. Liposomes formed by these methods however, are mechanically unstable in whole blood due to their high curvature and are rapidly removed from systemic circulation via low-density lipoprotein (LDL) exchange.

Extrusion techniques are the most widely used methods for SUV liposome production for *in vitro* and *in vivo* studies due to their ease of production, readily selectable particle diameters (dictated by the nominal pore size of the track-etch membranes used for extrusion, typically between 50-120 nm for *in vivo* experiments), batch-to-batch reproducibility, and freedom from solvent and/or surfactant contamination. Solvent injection and detergent dialysis techniques for liposome production give heterogeneous distributions of particle sizes and are not commonly used for biophysical or biochemical experimentation due to the retention of membrane impurities in these particles. Materials to be encapsulated may be passively entrapped or "remote" loaded.

Loading Drugs Into Liposomes

Several methods by which drugs are loaded into liposomes are described in Ostro and Cullis, Am. J. Hosp. Pharm. 456:1567-1587 (1989) and by Juliano, "Interactions of Proteins and Drugs with Liposomes," in *Liposomes*, Ibid., which are both incorporated by reference. Most drugs are loaded at the time the liposome is formed by co-solubilizing the drug with the starting materials. The site of the liposome (cavity or membrane) into which the drug is located depends on the properties of the drug. A hydrophobic drug such as amphotericin B, for example, is co-solubilized with lipid in an organic solvent. See

Lopez-Bernstein, J. Infect. Dis. 147:939-45 (1983). Subsequent removal of the solvent and subsequent hydration of the liposome yields a liposome drug complex with the hydrophobic drug primarily in the membrane.

Water soluble drugs can be sequestered in the liposome cavity by submitting liposomes to several cycles of freezing and thawing in an aqueous solution containing the drug, as described above under Liposomal Preparation. Finally, charged amphiphatic drugs can be loaded into preformed liposomes using transmembrane pH gradients, as described in Bally et al., Biochem. Biophys. Acta 812:66-76 (1985).

Despite many years of investigation, selective targeting and membrane translocation of compounds to cells in the body remains problematic. One limitation to the widespread use of liposomes derives from the rapid accumulation of intravenously administered liposomes in the reticuloendothelial system. Even with targeting entities bound to the liposome surface, liposomes accumulate rapidly in organs with fenestrated capillaries, such as the liver, spleen, and bone marrow. The uptake of liposomes by the reticuloendothelial system can be limited by the inclusion of glycolipids such as monosialoganglioside (GM1) or hydrogenated Phosphatidylinositol (HPI) in the lipid bilayer (Litzinger, D.C. and Huang, L. (1992) *Biochim. Biophycs. Acta*, 1104, 179-187). Alternatively a measurable fraction of the externally exposed lipids can be derivatized with polyethyleneglycol (PEG), see for example, Moghimi, S.M. and Patel, H.M. (1992) *Biochim Biophycs. Acta*, 1135, 269-274. The PEG coating is believed to inhibit nonspecific adsorption of serum proteins and thereby prevent nonspecific recognition of the liposomes by macrophages (Papähadjopoulos, D., Allen, T.M., Gabison, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.-D., Woodle,

M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) *Proc. Natl. Acad. Sci. USA*, 88, 11460-11464).

PEG derivatization is now commonly used to prevent liposome phagocytosis by the reticuloendothelial system. Such "stealth liposomes" are reported to survive more than 24 hours in circulation compared to only ~ 2 hours observed for their unprotected counterparts (Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P. and Huang, L. (1991) *Biochim Biophycs. Acta*, 1062, 142-148).

Although surface attached PEG groups inhibit the uptake by the reticuloendothelial system, PEG also interferes with the interaction/binding of any ligands present on the external surface of the liposome with their respective cellular targets. To overcome this inhibitory effect, the targeting ligands can be attached to the ends of the polymeric chains that render the liposomal resistant to uptake by the reticuloendothelial system (Kilanov, A.L., and Huang, L., Long Circulating Liposomes: Development and Perspectives, Journal of Liposome Research, 2(3), P. 321-334 (1992).

Once a liposome has been delivered to its target site the contents typically must be released to the cell cytoplasm to have their desired effect. Drug escape from liposomes localized within tumor interstitia or endosomal compartments, however, is often observed to be quite slow. In most cases, this results in the release of nontherapeutic/nonlethal drug concentrations or lysosomal drug degradation. Researchers have focused on ways to "trigger" the release liposome contents into the cytoplasm of the cells to enhance the speed and effective delivery of encapsulated exogenous molecules to the cytoplasm of cells.

One approach involves promoting leakage of liposome contents by heating a liposomal saturated target site above a critical temperature range, for example by

radio frequency heating of target tissues. Yatvin et al., Science 202:1290 (1978). Another approach has used liposomes prepared from pH sensitive lipids, which leak their pharmaceutical contents into low pH target regions. Such areas of localized acidity are sometimes found in tumors, hence it has been proposed that intravenous administration of such liposomes would selectively release anti-cancer chemotherapeutic agents at target tumors. Yatvin et al., Science 210:1253 (1980).

U.S. Pat. No. 4,882,164 similarly discloses a light sensitive liposome which undergoes a trans to cis isomerization upon irradiation with an appropriate wavelength of light (ultraviolet light) to allow the fluid contents of the liposome to escape through the membrane into the surrounding environment. Finally, GB Patent 2,209,468 discloses liposomes with an incorporated photosensitizing agent that absorbs light and alters the lipid membrane to release a drug from the liposome.

The development of liposomes that could be targeted to a population of cells and induced to release their payload upon activation by a metabolic or externally applied trigger would greatly improve the efficacy of liposomes as a delivery vehicle.

The present invention is directed to a novel composition, and method of using that novel composition, for improving the delivery of exogenous molecules to the cytoplasm of cells. The novel delivery system comprises an exogenous molecule entrapped by a liposome vesicle, wherein a targeting ligand is complexed (either directly or indirectly) to the surface of the liposome, and the liposome comprises a triggerable membrane fusion lipid.

Summary of the Invention

An improved liposome and method for delivering an exogenous molecule to the cytoplasm of a cell is described.

The liposomal membrane comprises triggerable lipids and lipids complexed to a ligand, wherein the ligand is capable of interacting with cellular membranes to enhance the uptake of the ligand and attached liposome. In accordance with one embodiment the triggerable lipid contains a vinyl ether functionality which is cleaved in response to a reduction in pH to produce a local disruption in the liposomal membrane.

Brief Description of the Drawings

Fig. 1: Graphic representation of the percent calcein released relative to the percent DP1sC hydrolyzed from DP1sC:DHC liposomes at pH 4.5 as a function of DHC content.

Fig. 2: Graphic representation of the percent calcein released per time from DP1sC:DHC liposomes at pH 4.5 as a function of DHC content.

Fig. 3: Graphic representation of the propidium iodide release kinetics in KB cells using folate-targeted DP1sC:DHC liposomes.

Fig. 4: Graphic representation of the release of PI from liposomal vessicles into the cytoplasm of cultured KB cells.

Fig. 5: Graphic representation of the cytotoxicity of arabinofuranosylcytocine (Ara-C) in KB cell cultures. Cells were plated to 50% confluence in 24-well culture plates before treatment with free Ara-C (diamonds), Ara-C encapsulated in EPC:folate liposomes (squares), or DP1sC:folate liposomes (triangles) for 4 h. The cells were then washed, incubated in fresh FDMEM, and analyzed for DNA synthesis after 24 h.

Fig. 6: Graphic representation of total PI bound to cultured KB cells after incubation of the cells with targeted and non-targeted PI encapsulated lyosomes.

Detailed Description of the Invention

Definitions

A triggerable lipid is defined herein as a lipid that undergoes a chemical or conformational change upon exposure to a predetermined condition.

A pH sensitive lipid is defined herein as a lipid that undergoes a chemical or conformational change upon exposure to a decreased pH.

The term "complexed" is used herein to designate a linkage between two entities through a covalent, ionic or hydrogen bond.

A targeting lipid is defined herein as a lipid ligand complex, wherein the ligand is capable of being internalized by receptor mediated uptake by the cell.

Actively and passively targeted liposomes have attracted a great deal of attention as drug delivery vehicles due to their favorable biocompatibility, high drug:lipid ratios, and blood clearance characteristics. Methods for efficiently, transporting the liposomal contents to the target cell cytoplasm, however, have not been generally available in the form of a plasma-stable liposome. This obstacle is especially problematic for the cytoplasmic delivery of peptides, antisense oligonucleotides, and gene constructs.

The present invention is directed to an improved liposome that enhances the delivery of exogenous molecules to the cytoplasm of a targeted population of cells. The enhanced delivery can be quantitated in terms of selectivity, speed of uptake, and as the percentage of material delivered to the cytoplasm. The hybrid liposome system of the present invention, obviates these problems by incorporating both ligand receptor-mediated targeting moieties and a cytoplasmic release mechanism. The ligand enhances the cellular uptake of the liposome by the

targeted cells and the cytoplasmic release mechanism (for example, vinyl ether-based triggerability upon exposure to the low pH environment of the endosome) enhances the delivery of exogenous molecules to the cytoplasm of cells.

In accordance with one embodiment of the present invention, phospholipids suitable for the formation of liposomes are modified by complexing a ligand to the phospholipid headgroup using techniques know to those skilled in the art. These modified lipids are combined with additional lipids, including triggerable lipids, to prepare a liposomal complex in accordance with the present invention.

In accordance with one embodiment, phospholipids suitable for the formation of liposomes are modified by covalently linking a spacer (for example, a PEG molecule) to the phospholipid headgroup and linking (through a covalent, ionic or hydrogen bond) the opposite end of the linker to a ligand, wherein the ligand is subject to receptor mediated cellular uptake. These modified lipids are combined with additional lipids, including for example, pH sensitive lipids such as diplasmenylcholine lipid (1,2-di-*O*-(Z-1'-hexadecenyl)-*sn*-glycero-3-phosphatidylcholine or DPlsC), to prepare a targeted liposomal complex in accordance with the present invention. The liposome complex is loaded with an exogenous molecule using methods known to those of ordinary skill in the art. Upon contact of the liposome complex with a cell membrane bearing a receptor associated with the ligand, receptor mediated transmembrane transport is initiated thus internalizing the complex within the cell.

Ligands useful in accordance with the present invention include any compound that mediates uptake of that compound by a cell. In one embodiment the ligand interacts with a particular cell type or tissue, and thus linking the ligand to the liposome enables the preferential uptake

(i.e. targeting) of liposomes by that particular cell type or tissue. Suitable ligands useful for mediating the uptake of a liposome include antibodies and/or compounds capable of binding to a receptor and being internalized by receptor mediated endocytosis.

Vitamins and other essential minerals and nutrients can be utilized to enhance the uptake of exogenous molecules. In particular a vitamin ligand can be selected from the group consisting of folate, folate receptor-binding analogs of folate, and other folate receptor-binding ligands, biotin, biotin receptor-binding analogs of biotin and other biotin receptor-binding ligands, riboflavin, riboflavin receptor-binding analogs of riboflavin and other riboflavin receptor-binding ligands, and thiamin, thiamin receptor-binding analogs of thiamin and other thiamin receptor-binding ligands. Additional nutrients believed to trigger receptor mediated endocytosis, and thus also having application in accordance with the presently disclosed method, are carnitine, inositol, lipoic acid, niacin, pantothenic acid, pyridoxal, and ascorbic acid, and the lipid soluble vitamins A, D, E and K. Furthermore any of the "immunoliposomes" (liposomes having an antibody linked to the surface of the liposome) described in the prior art are suitable for use in the present invention.

The liposomal carrier system of the present invention can be utilized to deliver a variety of exogenous molecules to the cytoplasm of cells, including diagnostic agents and molecules capable of modulating or otherwise modifying cell function, such as pharmaceutically active compounds. These compounds can be entrapped by the liposome vesicles of the present invention either by encapsulating water-soluble compounds in their aqueous cavities, or by carrying lipid soluble compounds within the membrane itself.

Exogenous molecules for use in accordance with the present invention can include, but are not limited to: peptides, oligopeptides, proteins, apoproteins, glycoproteins, antigens and antibodies thereto, haptens and antibodies thereto, receptors and other membrane proteins, retro-inverso oligopeptides, protein analogs in which at least one non-peptide linkage replaces a peptide linkage, enzymes, coenzymes, enzyme inhibitors, amino acids and their derivatives, hormones, lipids, phospholipids, liposomes; toxins such as aflatoxin, digoxin, xanthotoxin, rubratoxin; antibiotics such as cephalosporins, penicillin, and erythromycin; analgesics such as aspirin, ibuprofen, and acetaminophen, bronchodilators such theophylline and albuterol; beta-blockers such as propranolol, metoprolol, atenolol, labetolol, timolol, penbutolol, and pindolol; antimicrobial agents such as those described above and ciprofloxacin, cinoxacin, and norfloxacin; antihypertensive agents such as clonidine, methyldopa, prazosin, verapamil, nifedipine, captopril, and enalapril; cardiovascular agents including antiarrhythmics, cardiac glycosides, antianginals and vasodilators; central nervous system agents including stimulants, psychotropics, antimanics, and depressants; antiviral agents; antihistamines such as chlorpheniramine and brompheniramine; cancer drugs including chemotherapeutic agents such as saporin, Pseudomonas exotoxin, Diptheria toxin fragement A, Ara C, 5-Flourouracil, Taxol, cis platin, methotrexate, vincristine, doxorubincin, and vineblastin; tranquilizers such as diazepam, chordiazepoxide, oxazepam, alprazolam, and triazolam; anti-depressants such as fluoxetine, amitriptyline, nortriptyline, and imipramine; H-2 antagonists such as nizatidine, cimetidine, famotidine, and ranitidine; anticonvulsants; antinauseants; prostaglandins; muscle relaxants; anti-inflammatory substances; ; stimulants; decongestants; antiemetics; diuretics;

antispasmodics; antiasthmatics; anti-Parkinson agents; expectorants; cough suppressants; mucolytics; vitamins; and mineral and nutritional additives. Other molecules include nucleotides; oligonucleotides; polynucleotides; and their art-recognized and biologically functional analogs and derivatives including, for example; methylated polynucleotides and nucleotide analogs having phosphorothioate linkages; plasmids, cosmids, artificial chromosomes, other nucleic acid vectors; antisense polynucleotides including those substantially complementary to at least one endogenous nucleic acid or those having sequences with a sense opposed to at least portions of selected viral or retroviral genomes; promoters; enhancers; inhibitors; other ligands for regulating gene transcription and translation.

Overview of Liposome Triggering Mechanisms

Table 1 summarizes the various physical and chemical phenomena that can be used as a basis for liposome triggering. Many of these approaches have, in fact, been explored for unloading liposomes upon application of an external stimulus.

Table 1

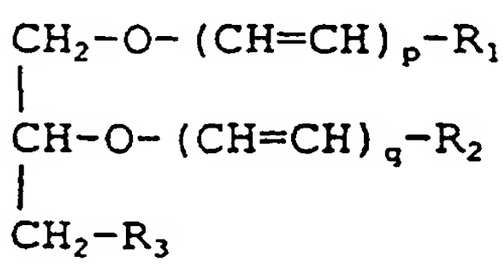
Liposome Triggering Methods
Chemical Transformations of Amphiphilic Molecules
Extrusion of N_2, CO_2, SO_2, NH_3, and other gases
Hydrolysis
Photodissociation
Photoisomerization
(Photo)oxidation
Photopolymerization
Redox-initiated ligand exchange
Supramolecular Activation Pathways
Deprotection of membrane lytic or fusion agent
Osmotic shock
Phase transition (chemically or thermally induced)
(Photo)acoustic shear
Photo)thermal stimulation (e.g., light, microwaves, bulk heating, etc.)
Polymer adsorption or solubility change

Progress in the area of triggered liposome release and membrane fusion has been hampered by poor understanding of the molecular mechanisms of membrane permeability, lipid phase transitions and bilayer-bilayer fusion. For example, aggregation and membrane-membrane contact, promoted either by polyvalent cations (e.g., Ca^{2+}), proteins, or lectins, are thought to be important first steps in liposome leakage and membrane fusion. Additional factors are clearly involved, though, since many aggregating liposomal systems show little or no propensity to undergo membrane fusion or content leakage.

Membrane fusion rates depend on both the molecular properties of the membrane bilayer (e.g., lipid headgroup charge, lateral mobility, and intrinsic curvature), as well as its supramolecular properties (e.g., hydration layer thickness, bilayer composition, membrane asymmetry, lateral phase separation, and thermally induced density fluctuations). Content leakage, on the other hand, is less well understood since the inherent leakage properties of a liposomal membrane will be dependent on the

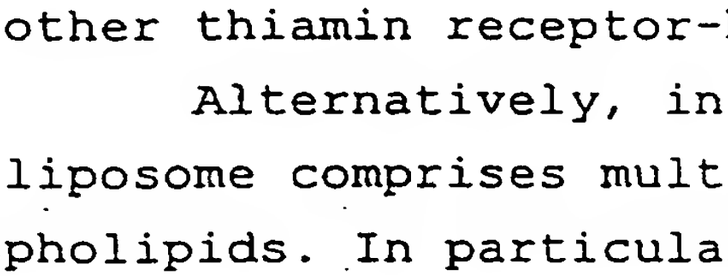
physical state and composition of the membrane bilayer, the presence of transient vs. persistent defects (pores) size and surface density of the defects, as well as the properties of the contents that are effusing from it.

In accordance with the present invention, a novel liposomal composition is provided for enhancing delivery of an exogenous molecule to the cytoplasm of a cell. The composition comprises a liposome, wherein said liposome membrane contains amphipathic lipids, preferably phospholipids, having a polar head group and two lipophilic chains that allow the lipid to pack into a bilayer structure. At least a portion of the phospholipids comprising the liposome membrane have lipophilic chains containing a vinyl ether functionality. In one preferred embodiment both lipophilic chains contain a vinyl ether functionality. A specific phospholipid (pH sensitive lipid) that fulfills this requirement is a plasmalogen having the formula:



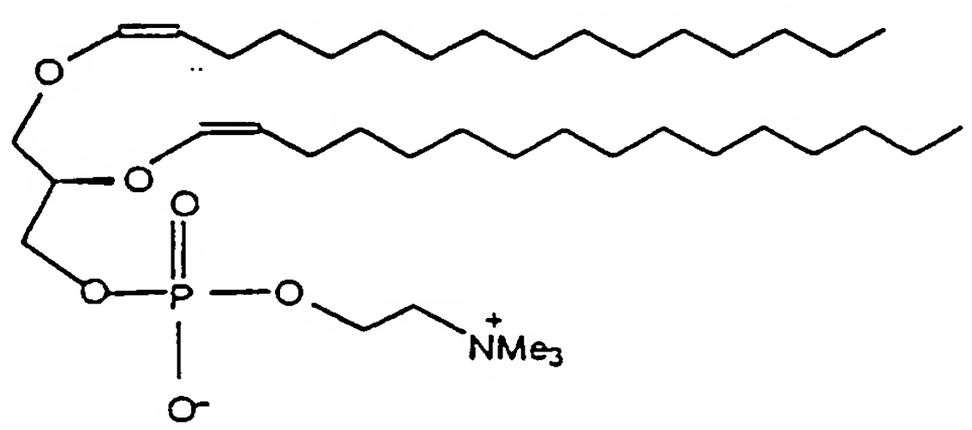
wherein p and q are independently 0 or 1 and at least p or q is 1, R_1 and R_2 are independently C_{12}-C_{24} alkyl or C_{12}-C_{24} alkenyl and R_3 is a bilayer forming phosphoryl ester of the formula $-CH_2OPO_2OR$, wherein R is selected from the group comprising 2-aminoethyl, 2-(trimethylamino)ethyl, 2-(N,N-dimethylamino)ethyl, 2-(trimethylammonium)ethyl, 2-carboxy-2-aminoethyl, succinamidoethyl, or inosityl. In one preferred embodiment, q and p are each 1, and R_1 and R_2 are each $(CH_2)_nCH_3$, where n is 12-24. In another preferred embodiment, one of R_1 or R_2 is 12-16 carbons long, and the other chain is at least 16 carbons long, more preferably 18 carbons.

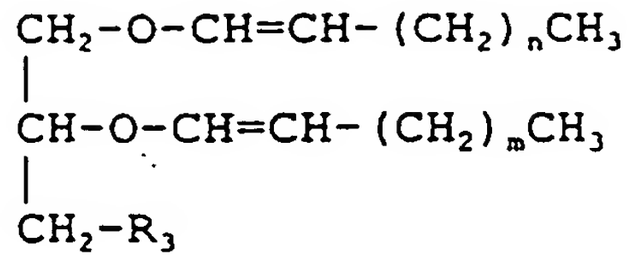
In accordance with one embodiment, a novel liposomal composition is provided for enhancing delivery of an exogenous molecule to the cytoplasm of a cell. The composition comprises an exogenous molecule encapsulated in a liposome, wherein said liposome comprises liposome-forming phospholipids, at least a portion of which are complexed to a ligand, and a portion of which comprise vinyl ether phospholipids of the formula:



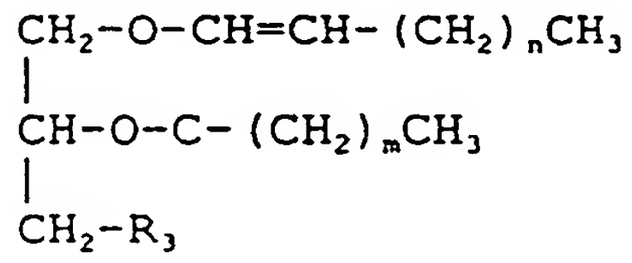
wherein R_3 is a phosphoryl ester and n and m are independently 12-24. Preferably the ligand of the phospholipid-ligand complexes is subject to receptor mediated cellular uptake, and in one embodiment the ligand is selected from the group consisting of folate, folate receptor-binding analogs of folate, and other folate receptor-binding ligands, biotin, biotin receptor-binding analogs of biotin and other biotin receptor-binding ligands, riboflavin, riboflavin receptor-binding analogs of riboflavin and other riboflavin receptor-binding ligands, and thiamin, thiamin receptor-binding analogs of thiamin and other thiamin receptor-binding ligands.

Alternatively, in accordance with one embodiment the liposome comprises multiple types of vinyl ether phospholipids. In particular, in one embodiment the liposome comprises a vinyl ether phospholipid of the formula:





and a vinyl ether phospholipid of the formula:



wherein R_3 is a phosphoryl ester and n and m are independently 12-24.

In one embodiment in accordance with the present invention a plasma-stable liposome is formed comprising a naturally-occurring vinyl ether linked phospholipid, diplasmenylcholine (1,2-di-*O*-(Z-1'-hexadecenyl)-*sn*-glycero-3-phosphatidylcholine or DPlsC).

Acid-catalyzed hydrolysis of DPlsC liposomes produces glycerophosphatidylcholine, fatty acids and aldehydes, and permeability of the liposome membrane increases significantly when ≥20% of the DPlsC lipids are hydrolyzed. Unlike many pH-sensitive liposome formulations, DPlsC liposomes possess remarkable plasma stability characteristics at 37°C and neutral pH. Pure DPlsC liposomes do not leak calcein upon exposure to 10% heat-inactivated fetal calf serum (HIFC) for up to 48 h. Pure DPlsC liposomes did leak 27% and 33% of encapsulated calcein upon exposure to 50% HIFC for 24 or 48 h, respectively. However, the addition of ≥10% dihydrocholesterol (DHC) to the DPlsC membrane is sufficient to stabilize the liposomes in 50% HIFCS for up to 48 h (See Table 2). These results suggest that DPlsC liposomes are sufficiently plasma-stable for drug delivery and transfection applications.

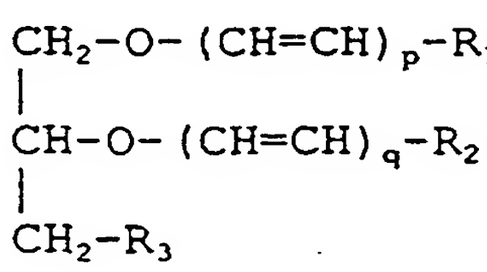
TABLE 2.

Liposome Stability at pH 7.4, 37°C

Liposome Type	[a]50% serum 24 hrs	[a]50% serum 48 hrs	[b]10% serum 48 hrs
DPlsC + no DHC	27%	33%	0
DPlsC + 10% DHC	0	0	0
DPlsC + 20% DHC	0	0	0
DPlsC + 30% DHC	0	0	0
DPlsC + 40% DHC	0	0	0

[a, b] Liposomes were mixed with pure heat-inactivated fetal calf serum at 1:1 and 9:1 ratios, respectively. % calcein release values are ± 5%.

The liposomes of the present invention are utilized in an improved method for delivering an exogenous molecule to the cytoplasm of a targeted living cell. This method can be performed either *in vivo* or *in vitro*. The method comprises the step of contacting a cell with a liposome complex, wherein the complex includes a liposome, having the exogenous molecule encapsulated therein. The liposome itself has ligands associated with its exterior surface and the liposome comprises a pH sensitive lipid having the formula:

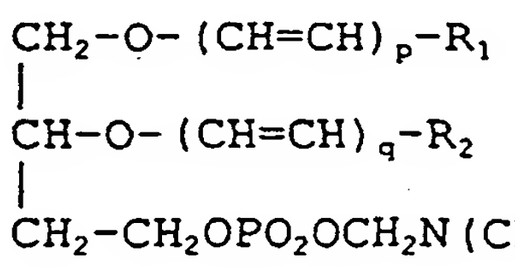


wherein p and q are independently 0 or 1 and at least p or q is 1, R_1 and R_2 are C_{12}-C_{24} alkyl and R_3 is a bilayer forming phosphoryl ester of the formula $-CH_2OPO_2OR$, wherein R is selected from the group comprising 2-aminoethyl, 2-(trimethylamino)ethyl, 2-(N,N-dimethylamino)ethyl, 2-(trimethylammonium)ethyl, 2-carboxy-2-aminoethyl, succinamidoethyl, or inosityl. In one preferred

embodiment, q and p are each 1, and R_1 and R_2 are each $(CH_2)_nCH_3$, where n is 12-24. In another preferred embodiment, one of R_1 or R_2 is 12-16 carbons long, and the other chain is at least 16 carbons long, more preferably 18 carbons.

The ligand associated with the surface of the liposome is preferably linked to the phospholipid headgroups via covalent, ionic or hydrogen bonds and the ligand is selected from the group consisting of folate, folate receptor-binding analogs of folate, and other folate receptor-binding ligands, biotin, biotin receptor-binding analogs of biotin and other biotin receptor-binding ligands, riboflavin, riboflavin receptor-binding analogs of riboflavin and other riboflavin receptor-binding ligands, and thiamin, thiamin receptor-binding analogs of thiamin and other thiamin receptor-binding ligands.

In one embodiment the liposome complex comprises a liposome encapsulating an exogenous molecule, wherein the liposome comprises a targeting lipid and a pH sensitive lipid having the formula:



wherein p and q are independently 0 or 1 and at least p or q is 1, R_1 and R_2 are C_{12}-C_{24} alkyl, and a lipid covalently linked to a ligand. The targeting lipid, in accordance with one embodiment, is a lipid of the formula DSPE-linker-ligand and one preferred linker is a polyethyleneglycol spacer arm. Typically the liposome comprises about 0.1% to about 1.5% of the targeting lipid, about 20% to about 99.5% of the pH sensitive lipid with the remainder being any amphipathic lipid having a polar head group and two lipophilic chains that allow the lipid to pack into a bilayer structure.

In one embodiment the liposome carrier comprises the pH sensitive lipid DP1sC, and a DSPE-PEG3350-folate conjugate (DSPE = distearoylphosphatidylethanolamine) for triggering and targeting of the liposome, respectively. The liposome optimally comprises about 0.1% to about 1.5%, more preferably about 0.1% to about 0.5%, DSPE-PEG3350-folate, about 60% to about 99.5%, more preferably about 80% to about 99.5% DplsC, and 0 to about 20%, more preferably about 10% or less, DHC.

Living cells which can serve as the target for the method of this invention include prokaryotes and eukaryotes, including yeasts, plant cells and animal cells. The present method can be used to modify cellular function of living cells *in vitro*, i.e., in cell culture, or *in vivo*, where the cells form part of, or otherwise exist in plant tissue or animal tissue. Exogenous molecules encapsulated within the disclosed liposomal delivery vehicles can be used to deliver effective amounts of diagnostic, pharmaceutically active, or therapeutic agents through parenteral or oral routes of administration to human or animal hosts. The present method can be performed on any cells in any manner which promotes contact of the liposome complex with the targeted cells having the requisite receptors.

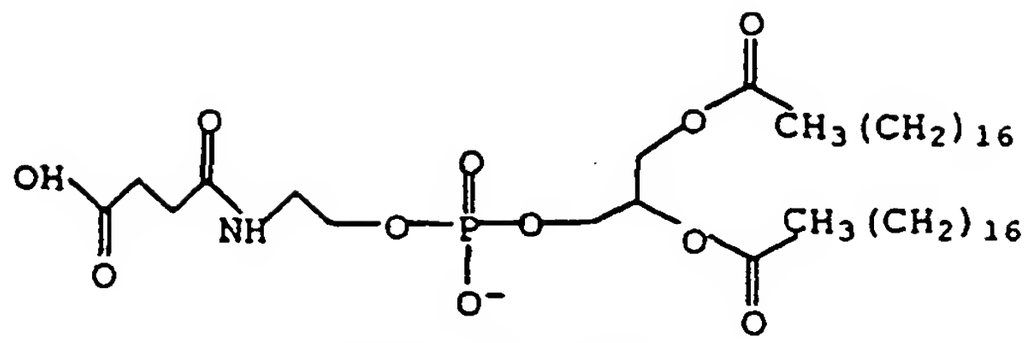
The liposomal compositions can be administered generally to an animal or human to target cells that form part of the tissue of the animal or human. Thus the target cells can include, for example, the cells lining the alimentary canal, such as the oral and pharyngeal mucosa, the cells forming the villi of the small intestine, or the cells lining the large intestine. Such cells of the alimentary canal can be targeted in accordance with this invention by oral administration of a composition comprising an exogenous molecule encapsulated by the liposome of the present invention. Similarly, cells lining

the respiratory system (nasal passages/lungs) of an animal can be targeted by inhalation of the present compositions; dermal/epidermal cells and cells of the vagina and rectum can be targeted by topical application of the present compositions; and cells of internal organs including cells of the placenta and the so-called blood/brain barrier can be targeted particularly by parenteral administration of the present compositions. Pharmaceutical formulations for therapeutic use in accordance with this invention contain effective amounts of the exogenous molecule encapsulated in the presently described liposomes, admixed with art-recognized excipients and pharmaceutically acceptable carriers appropriate to the contemplated route of administration.

Example 1

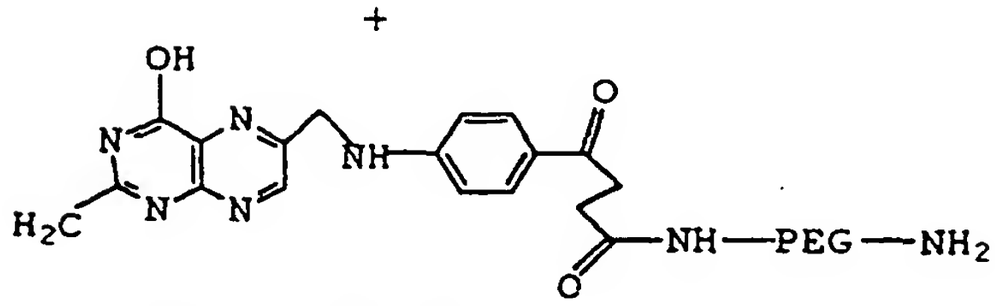
Synthesis of folate-PEG-DSPE.

The synthesis of the folate-PEG-DSPE construct is illustrated in accordance with Scheme I, shown below:



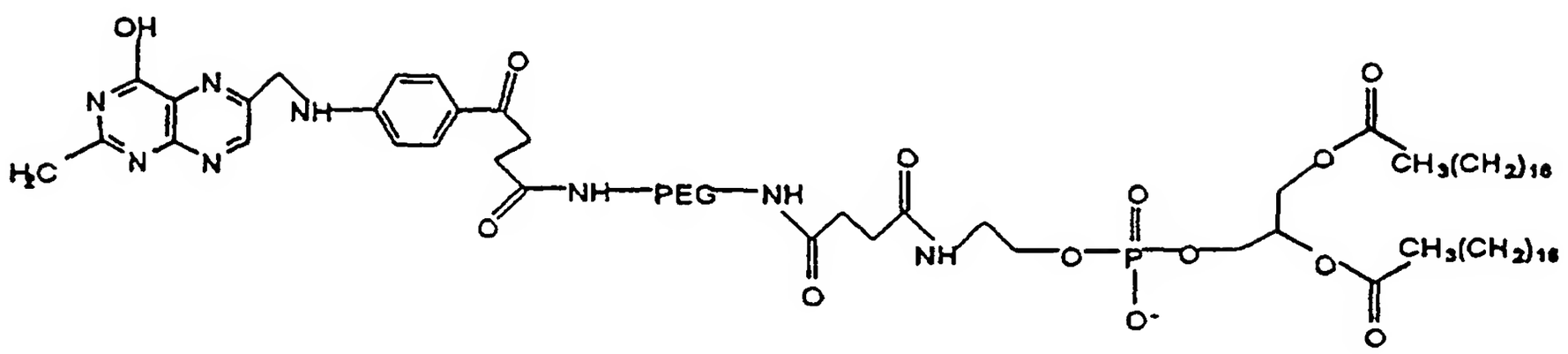
N-Succinyl DSPE

+



Folate-PEG-NH_2

DCC,
pyridine



Folate PEG DSPE

Folate-PEG-NH_2 was synthesized by reacting 500 mg polyoxyethylene-*bis*-amine with an equimolar quantity of

folic acid in 5 ml dimethylsulfoxide containing one molar equivalent of dicyclohexycarbodiimide and 10 µl pyridine. The reaction mixture was stirred overnight in the dark at room temperature. At this point, 10 ml water was added and the insoluble by-product, dicyclohezylurea, was removed by centrifugation. The supernatant was then dialyzed against 5 mM $NaHCO_3$ buffer (pH 9.0) and then against deionized water to remove the dimethylsulfoxide and unreacted folic acid in the mixture. The trace amount of unreacted polyoxyethylene-*bis*-amine was then removed by batch-adsorption with 5 g of cellulose phosphate cation-exchange resin pre-washed with excess 5 mM phosphate buffer (pH 7.0). Although not necessary, the trace amount of PEG-*bis*-folate may be removed by anion-exchange chromatography on a DEAE-trisacryl Sepharose column. Folate-PEG-amine can be easily eluted with 10 mM NH_4HCO_3 (pH 8.0). The produced folate-PEG-NH_2 was then lyophilized and analyzed for folate content by absorbance at 363 nm and -NH_2 content by the ninhydrin assay. The ratio of folate to free -NH_2 groups in this product was ≈1.

N-Succinyl-DSE was synthesized by reacting overnight 1.1 molar equivalent of succinic anhydride with 100 mg DSPE in 5ml chloroform containing 10 µl pyridine. The product was precipitated with cold acetone and verified by thin-layer chromatography. N-Succinyl-DSPE was re-dissolved in chloroform and its carboxyl group was activated by reacting with one molar equivalent of dicyclohexyl-carbodiimide for 4 h at room temperature. An equimolar amount of the above synthesized folate-PEG-NH_2 dissolved in chloroform was then added. After overnight stirring at room temperature, the solvent was removed from the reaction mixture, and the lipid pellet containing the folate-PEG-DSPE conjugate was washed twice with cold acetone, redissolved in chloroform, and stored at -20°C. The formation of folate-PEG-DSPE was confirmed by reverse-

phase high-pressure liquid chromatography.

Preparation of folate targeted dihydrocholesterol-free liposomes (DPlsC:Folate):

Diplasmenylcholine (DPlsC) lipid was prepared as described in Rui and Thompson, The Journal of Organic Chemistry 59, pp. 5758-5762 (1994) the disclosure of which is expressly incorporated herein. 13.6 mg of DPlsC was dissolved in 0.5 ml $CHCl_3$ and 15 µl of folate-PEG-DSPE conjugate solution (6.7 mM in $CHCl_3$) was added. The mixture was evaporated with a stream of dry N_2 to form a thin lipid film; this film was evaporated further by lyophilization for 3 hours in a 1 µ vacuum. The dried thin film was then hydrated with 1.0 ml of propidium iodide solution (10 mg/ml in pH 7.4 HEPES buffer containing 150 mM NaCl) using five freeze-thaw-vortex cycles to disperse the lipid as multilamellar liposomes (MLV). The MLV were extruded 10 times through two stacked 0.1 µm polycarbonate membranes at 55 °C. The unencapsulated propidium, iodide was removed by gel chromatography using a Sephadex G-50 column and HEPES buffer, pH 7.4 as eluent.

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Example 2

Endosomal Release of Folate-Targeted Liposomes.

Cell Culture.

KB cells, a human nasopharyngeal epidermal carcinoma cell line were maintained in a medium containing physiological concentrations of folate, i.e., minimum essential medium minus the folic acid additives and supplemented with 10% heat-inactivated fetal calf serum. The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The folate content of the fetal calf serum supplement brings the folate concentration of the medium to a near physiological value for human serum.

Liposome preparation.

DP1sC Liposomes were prepared by hydration of thin lipid films in the presence of analyte (50mM calcein solution or 10 mg/ml propidium iodide in phosphate buffered saline), followed by extrusion at 55°C through two 100nm Nuclepore filters. Extraliposomal analytes were removed by Sephadex G-50 gel filtration. Calcein fluorescence dequenching was monitored by diluting 50 µl aliquots of the hydrolysis mixtuwre into 2 ml of 150 mM NaCl/20 mM HEPES, pH 7.4 prior to measurement of the calcein fluorescence spectrum; leackage rates were determined using a ratio method described below (under the heading: Assay). Folate-targeted DP1sC liposomes were prepared as descrived above, except that 0.5% DSPE-PEG3350-folate was incorporated in the lipid film prior to hydration in the presence of 10 mg/ml propidium iodide (PI). Extraliposomal propidium iodide was removed by gel filtration using 20 mM pphosphate buffered saline, pH 7.4 (PBS) as eluent.

Sample preparation for folate targeted liposomes containing 10% dihydracholesterol (DHC) (9:1 DPlsC:DHC:Folate):

432 µl of DHC solution (2 mg/ml in $CHCl_3$) and 15 µl of folate-PEG-DSPE conjugate solution (6.7 mM in $CHCl_3$) were added to 14.0 mg of DPIsC lipid. Liposomes were then prepared using the same procedure as described for Example 1 above.

Sample preparation for folate targeted liposomes containing 20% DHC (8:2 DPlsC:DHC Folate):

1.0 ml of DHC solution (2 mg/ml in $CHCl_3$) and 15 µl of folate-PEG-DSPE conjugate solution were added to 14.0 mg of DPIsC lipid. Liposomes were then prepared using the same procedure as described for #1 above.

Assay

To quantitate the intracellular release of contents from DPslC:folate liposomes, KB cells in FDMEM were incubated for 4 h at 37°C with DPslC:folate liposomes containing 5µM propidium iodide. The cells were then washed and incubated with fresh FDMEM for the desired time and then released from their culture dishes by incubation with 0.5mL of non-enzymatic cell dissociation solution (Sigma) for 15 min. After gently resuspending in 1.5mL of FDMEM, cell-associated fluorescence was measured on a Perkin Elmer MPF-44 A fluorescence spectrophotometer (Ex=540nm, Em=615nm). Minor levels of light scattering and autofluorescence were subtracted from the measured propidium iodide signal. After each measurement, the cell suspension was sonicated in an ice-water bath for 15-20 min to determine the fluorescence of maximum propidium iodide release. The percent of propidium iodide release was calculated according to the following equation: % release = $(flu_1 - flu_{initial}/flu_{max} - flu_{initial})$ 100, where flu, was the

fluorescence at each time point, and flu_{max} was the fluorescence of maximum release at the same time point. To directly visualize these results, a second set of KB cells in FDMEM were incubated and washed in the same manner, and examined with an Olympus BH-2-fluorescence microscope. Endosomal acidification inhibition control experiments were performed in the same manner, except that 25μM monensin or 50μM chloroquine (final medium concentrations) were maintained during the incubation in PBS and FDMEM.

Results:

Fluorescence assay of KB cells treated with DPlsC:folate liposomes containing encapsulated propidium iodide (PI) indicate that acidification of these folate-targeted liposomes within the endosomal compartment leads to rapid and efficient release of PI into the cytoplasm (83% PI release within 8 h). The ability of folate-targeted DPlsC:DHC liposomes to promote endosomal release in KB cells was evaluated by fluorometric assay (540 nm excitation, 615 nm emission) using PI as a fluorescent probe. PI fluorescence (λ_{ex}=540nm, λ_{cm}=615nm) increases approximately 50-fold upon binding to RNA or DNA. This property makes it especially effective in endosomal release asays, since a fluorescent signal from cell-internalized PI effctively arises only after it has escaped from the endosome into the cytoplasm. Endosomal unloading of PI was also confirmed by fluorescence microscopy. The intense nucleoli and cytoplasmic staining observed indicated that PI is effectively released within the cytoplasm.

No detectable calcein release occurs from DPlsC liposomes maintained at pH 7.4, 37°C for 48 h, in contrast to their leakage properties at pH 4.5 wherein the half-time for release ($t_{50\%}$ release) is 76 minutes. Calcein leakage rates increase with decreasing pH (Table 3) and with the

extent of DPlsC hydrolysis at pH 4.5 (Figure 1), however, they decrease with increasing mole fraction of the satureated cholesterol derivative, 5α-cholestane-8β-ol (dihyderocholesterol, DHC) (Figure 2).

TABLE 3

pH Dependendce on 50% Release Time

pH	$t_{50\% \text{ Release}}$ (min)
2.3	1.5
3.2	3.6
4.5	76
5.3	230
6.3	1740

Furthermore the cytoplasmic release of PI into the KB cells occured at a much greater rate from DPlsC:folate liposomes than from the non-triggerable liposome DPPC:folates (DPPC = 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine)(See Fig. 4).

Hydrolysis rates of DPlsC, monitoried by HPLC-ELS analysis, suggest that a critical extent of diplasmenylcholine degradation is required before the onset of rapid calcein leakage occurs, approximately 5-60% hydrolysis, depending on DHC content; Figure 1). DPlsC hydrolysis kinetics at pH 4.5, a pH regime that occurs within the endosomes of KB cells, are pseudo-first order ($k_{obs}=6.3x10^{-5}$ s^{-1} at pH 4.5). Calcein release rates, however, are non-linear, with dramatic increases in leakage rate occuring after a threshold level of lipid has been hydrolyzed. These results suggest that membrane destabilization occurs only after a critical concentration

of diplasmenycholine degradation products have accumulated within the bilayer.

PI release kinetics revealed that 83% of the encapsulated PI escaped both the liposomal and endosomal compartments within 8 hours when ≤10 mol% DHC was present in the DPlsC membrane; 36% release occurred within 8 h (50% after 24 h) when the DHC content was increased to 20 mol% (Fig. 3). Both the extent and rate of PI release were greater for DPlsC liposomes than for folate-targeted egg phosphatidyleholine (EPC) vesicles containing the pH-sensitive peptide EALA either covalently-attached (9% release in 8 h; 20% in 24 h) or added to the external medium (4% release in 8 h; 13% in 24 h). EALA is a 30 amino acid peptide of the sequence, AALAEALAEALAEALAEALAEALAAAAGC, that facilitates release of liposomal contents upon exposure to mildly acidic pH, see Vogel et al. J. Am. Chem Soc. 1995. Control experiments, using KB cells treated with PI encapsulated DPlsC:folate liposomes in the presence of the endosomal acidification inhibitors monensin (25μM) and chloroquine (50μM), indicated that <5% PI escaped into the cytoplasm when monitored for up to 24 hours aftger liposomal treatment. These results strongly suggest that an acidic endosomal compartment is necessary to trigger cytoplasmic content delivery from DPlsC liposomes.

Example 3

Sample preparation for cytotoxicity testing of Ara-C-containing DPlsC:folate Liposomes:

DPlsC (33.4 mg in 2.0 ml $CHCl_3$) was combined with 35 μl of folate-PEG-DSPE conjugate solution (6.7 mM in $CHCl_3$). The mixture was evaporated with a stream of dry N_2, the resulting thin film was lyophilized in a 1μ vacuum for

4 hours. The lipid film was then hydrated with 1.0 ml of Ara-C solution (2.0 M in pH 7.4 PBS buffer) for 4 hours, freeze-thaw-vortexed five times, and extruded 10 times through two stacked 0.1 μm polycarbonate membranes at 55°C. The extravesicular Ara-C was removed by gel filtration using a Sephadex G-50 column and phosphate buffered saline (PBS), pH 7.4 as eluent. The same procedure as described immediately above was used to prepare the control empty DPlsC:folate liposomes, except that the lipid was hydrated with PBS buffer containing no Ara-C.

Ara-C cytotoxicity assay:

KB cells were plated in 24-well culture plates and grown for 24 h to approximately 50% confluence before treatment with free Ara-C, Ara-C encapsulated in egg phosphatidylcholine (EPC):folate liposomes, and Ara-C encapsulated in DPlsC:folate liposomes. Liposomes were prepared as described in Example 2 except the lipids were hydrated in an Ara-C solution (PBS, pH 7.4); drug concentration after gel filtration=500 μM yielding a drug: lipid concentration ratio of 1:65. The liposomes were added to the KB cells and incubated for 4 h. The cells were then washed to remove the unbound drug and incubated in fresh media in the presence of 2μCi/well [^{3}H]thymidine. After 24 h, cells were lysed, and the DNA precipitated with trichloroacetic acid. The DNA was then dissolved in 2 N NaOH and the [^{3}H]thymidine incorporation measured by scintillation counting.

Results

The ability of folate-targeted DPlsC liposomes to trigger cytoplasmic delivery of Ara-C upon endosomal acidification was monitored by [3H]thymidine incorporation assay as described above. The results are summarized in Fig. 4, wherein cells were treated with free Ara-C

(diamonds), Ara-C encapsulated in EPC:folate liposomes (squares), or DP1sC:folate liposomes (triangles) for 4 h. The cells were then washed, incubated in fresh FDMEM, and analyzed for DNA synthesis after 24 h.

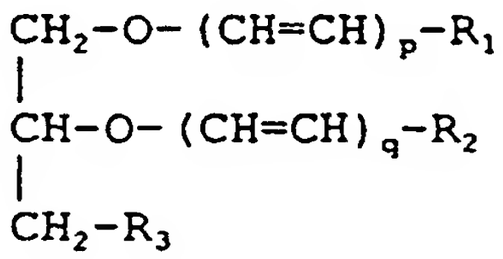
The IC_{50} value of Ara-C encapsulated in DP1sC:folate liposomes is 0.49 μM in KB cell cultures compared to an IC_{50} value of 2.6mM for free Ara-C. Thus folate-targeted DP1sC liposomes exhibit a remarkable 6000-fold enhancement of inhibition relative to free Ara-C in KB cell cultures. The IC_{50} value of Ara-C encapsulated in EPC:folate liposomes is 40.0μM in KB cell cultures, thus DP1sC:folate liposomes exhibit an approximate 100-fold enhancement over non-triggerable targeted liposomes. Furthermore, DP1sC:10 mol%DHC-folate liposomes containing Ara-C represent an improvement over transferrin-conjugated, Ara-C containing pH-sensitive PE liposomes by a factor of greater than sixty (the IC_{50} value for the transferin-liposomes is 30.0μM) and pH-sensitive immunoliposomes by a factor exceeding 1000. No inhibition of DNA synthesis was observed in KB cells treated with empty DP1sC-folate liposomes (control), indicating that neither the lipid nor its degradation products have a significant effect on cellular function at the lipid concentrations used. These results clearly demonstrate that pH triggering with DP1sC liposomes is a practical, fast, and efficient method for intracellular delivery of biologically active materials.

Fig. 6 shows total PI bound to KB cells. After KB cells were incubated with free PI or the various targeted (DP1sC:folate + DOPC:folate) and non-targeted (DP1sC) liposomes the cells were wshed and then lysed to determined the total ng PI bound to the cells. The data shows a significant increse in the numer of targeted liposomes bound to the KB cells relative to non-targeted liposomes and free PI.

Claims:

1. A composition for enhancing delivery of an exogenous molecule to the cytoplasm of a cell, said composition comprising;

a liposome, wherein said liposome comprises liposome-forming phospholipids, at least a portion of which are complexed to a ligand, and a portion of which comprise vinyl ether phospholipids of the formula:



wherein p and q are independently 0 or 1 and at least p or q is 1, R_1 and R_2 are independently C_{12}-C_{24} alkyl and R_3 is a phosphoryl ester;

and an exogenous molecule encapsulated by said liposome.

2. The composition of claim 1 wherein the ligand is covalently bound through the headgroup of said phospholipids.

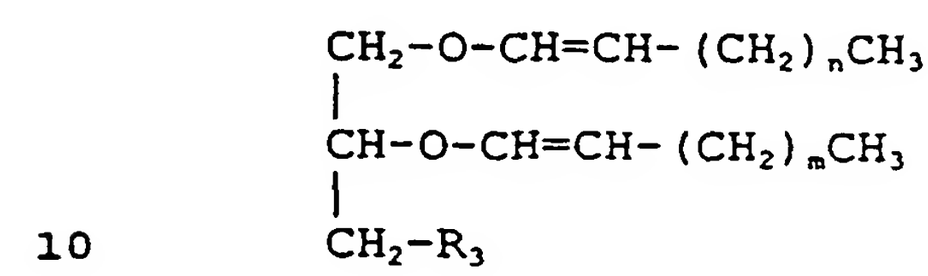
3. The composition of claim 1, wherein the ligand is complexed to the phospholipids via a linker.

4. The composition of claim 1 wherein the ligand is selected from the group consisting of folate, folate receptor-binding analogs of folate, and other folate receptor-binding ligands, biotin, biotin receptor-binding analogs of biotin and other biotin receptor-binding ligands, riboflavin, riboflavin receptor-binding analogs of riboflavin and other riboflavin receptor-binding ligands, and thiamin, thiamin receptor-binding analogs of thiamin and other thiamin receptor-binding ligands.

5. The composition of claim 3 wherein the ligand is selected from the group consisting of folate, folate

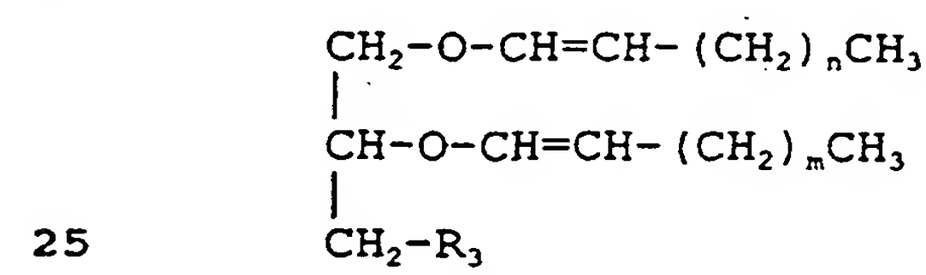
receptor-binding analogs of folate, and other folate receptor-binding ligands.

6. The composition of claim 1, wherein the vinyl ether phospholipid is a compound of the formula

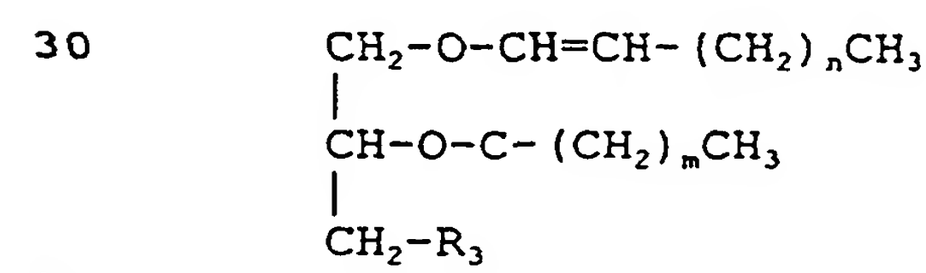


wherein R_3 is a phosphoryl ester of the formula $-CH_2OPO_2OR$, wherein R is selected from the group comprising 2-aminoethyl, 2-(trimethylamino)ethyl, 2-(N,N-dimethylamino)ethyl, 2-(trimethylammonium)ethyl, 2-carboxy-2-aminoethyl, succinamidoethyl, or inosityl, and n and m are independently 12-24.

7. The composition of claim 1, wherein the liposome comprises a vinyl ether phospholipid of the formula:



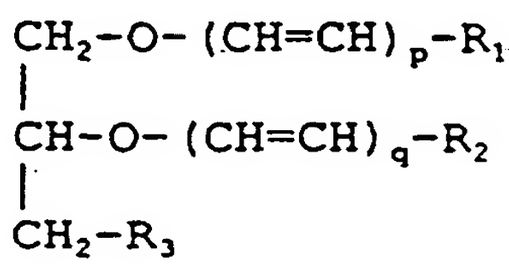
and a vinyl ether phospholipid of the formula:



wherein R_3 is a phosphoryl ester and n and m are independently 12-24.

8. An improved method for deliverying an exogenous molecule to the cytoplasm of a targeted living cell, the method comprising the step of contacting the cell with a liposome complex, said complex including a liposome having the exogenous molecule encapsulated therein, said liposome

bearing ligands associated with the liposome exterior membrane surface, said liposome further comprising a pH sensitive lipid having the formula:

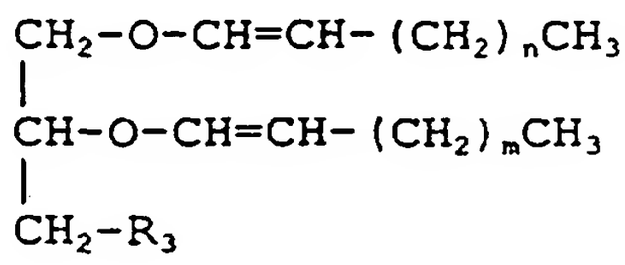


wherein p and q are independently 0 or 1 and at least p or q is 1, R_1 and R_2 are C_{12}-C_{24} alkyl and R_3 is a phosphoryl ester.

9. A liposome complex comprising

an exogenous molecule;

a liposome encapsulating said exogenous molecule therein, said liposome comprising a pH sensitive lipid having the formula:



wherein R_3 is phosphoryl ester and n and m are independently 12-24; and

a targeting lipid of the formula DSPE-linker-ligand.

10. The liposome complex of claim 9 wherein the linker is a polyethyleneglycol spacer arm.

11. The liposome complex of claim 9 wherein the ligand is selected from the group consisting of folate, folate receptor-binding analogs of folate, and other folate receptor-binding ligands, biotin, biotin receptor-binding analogs of biotin and other biotin receptor-binding ligands, riboflavin, riboflavin receptor-binding analogs of riboflavin and other riboflavin receptor-binding ligands, and thiamin, thiamin receptor-binding analogs of thiamin and other thiamin receptor-binding ligands.

12. The liposome complex of claim 9 wherein the pH sensitive lipid is DPlsC.

13. The liposome complex of claim 9 wherein about 0.1 to about 0.5% of the lipids forming said liposome are targeting lipids.

14. The liposome complex of claim 9 wherein about 80% to about 99.5% of the lipids forming said liposome comprise pH sensitive lipids.

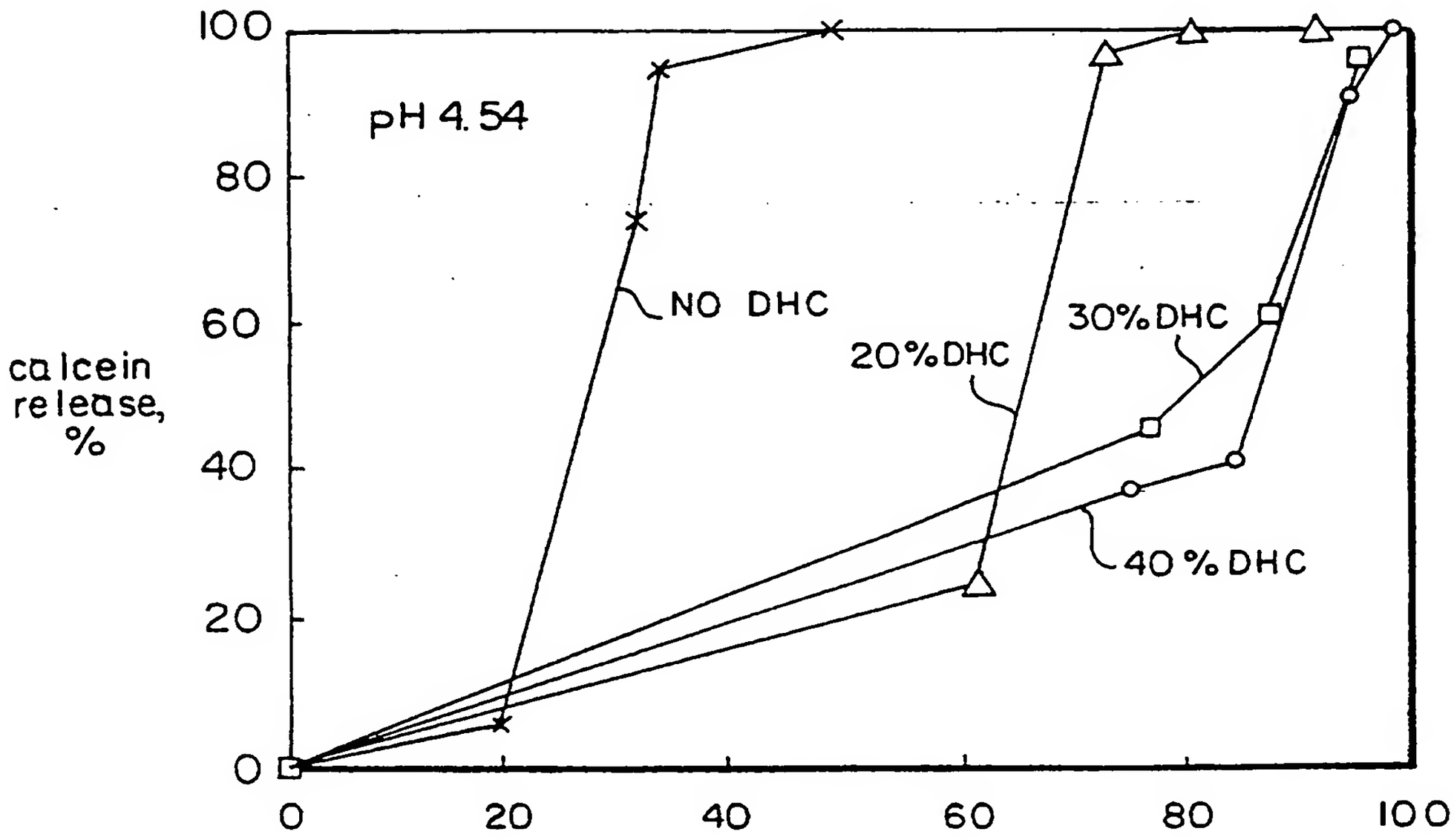

100
80
60
40
20
0
calcein release, %
pH 4.54
NO DHC
20% DHC
30% DHC
40% DHC
0
20
40
60
80
100
DplsC hydrolysis, %

FIG. 1

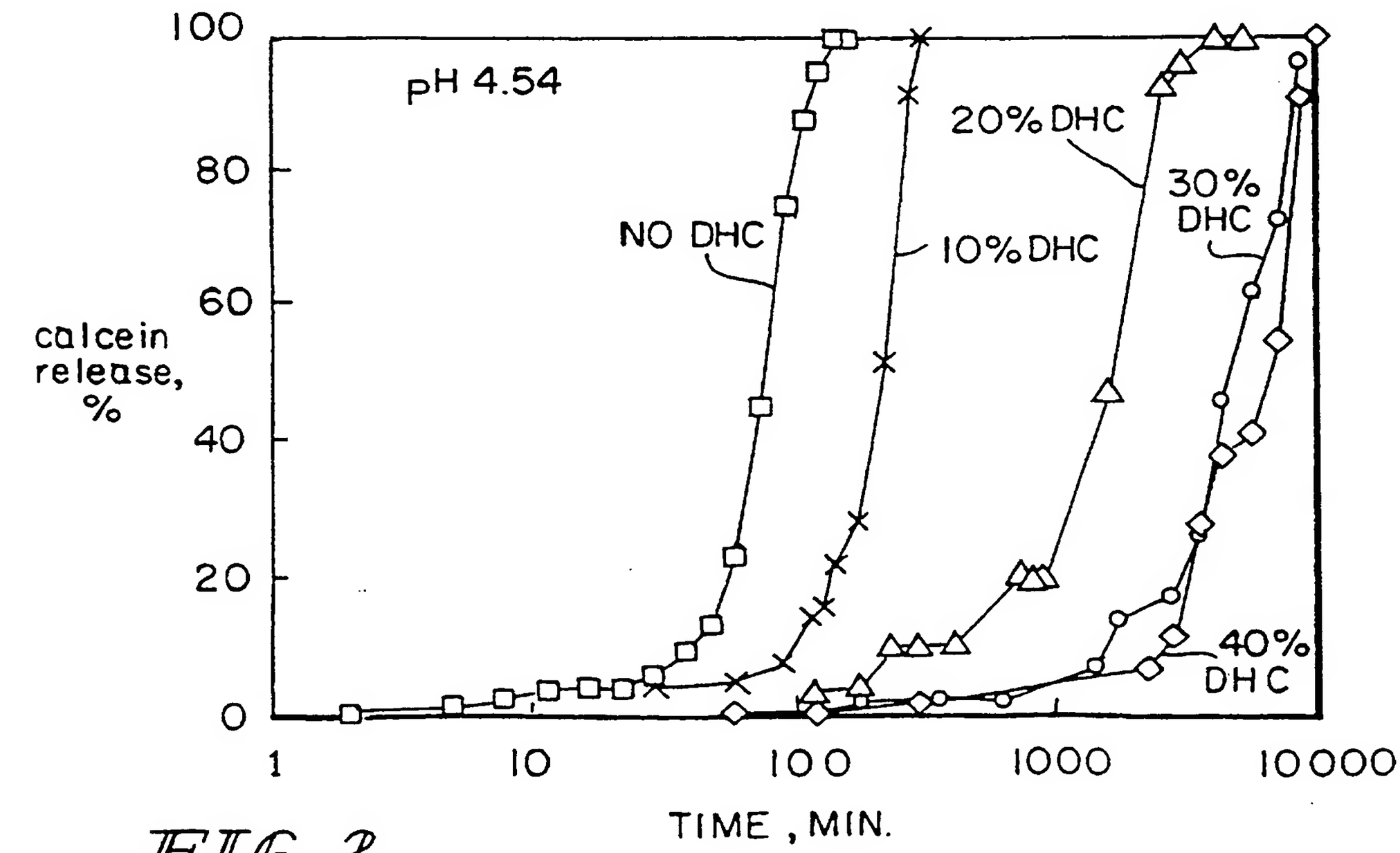

100
80
60
40
20
0
calcein release, %
pH 4.54
NO DHC
10% DHC
20% DHC
30% DHC
40% DHC
1
10
100
1000
10000
TIME, MIN.

FIG. 2

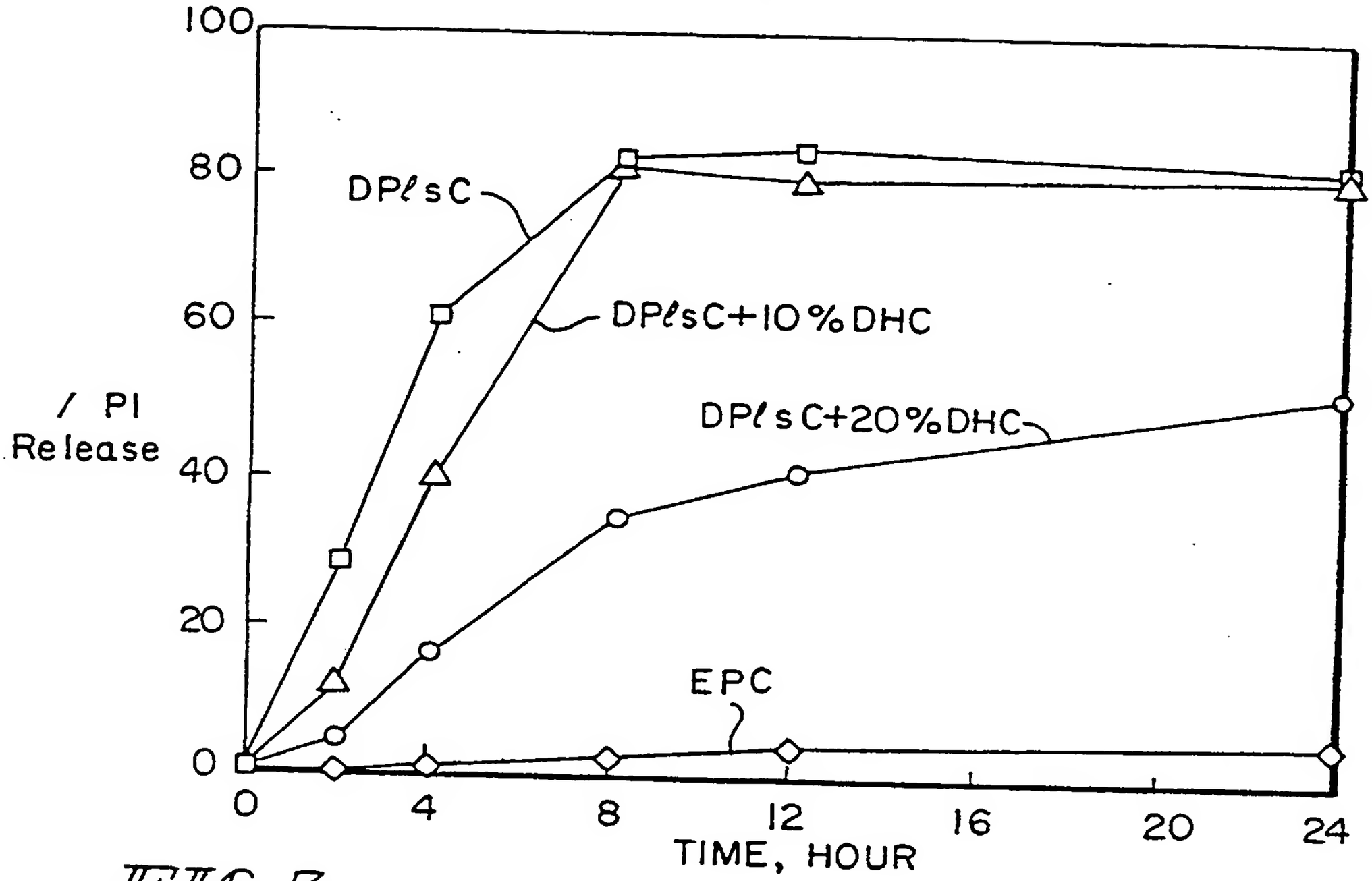

100
80
60
40
20
0
% PI Release
DPℓsC
DPℓsC+10% DHC
DPℓsC+20% DHC
EPC
0
4
8
12
16
20
24
TIME, HOUR

FIG. 3

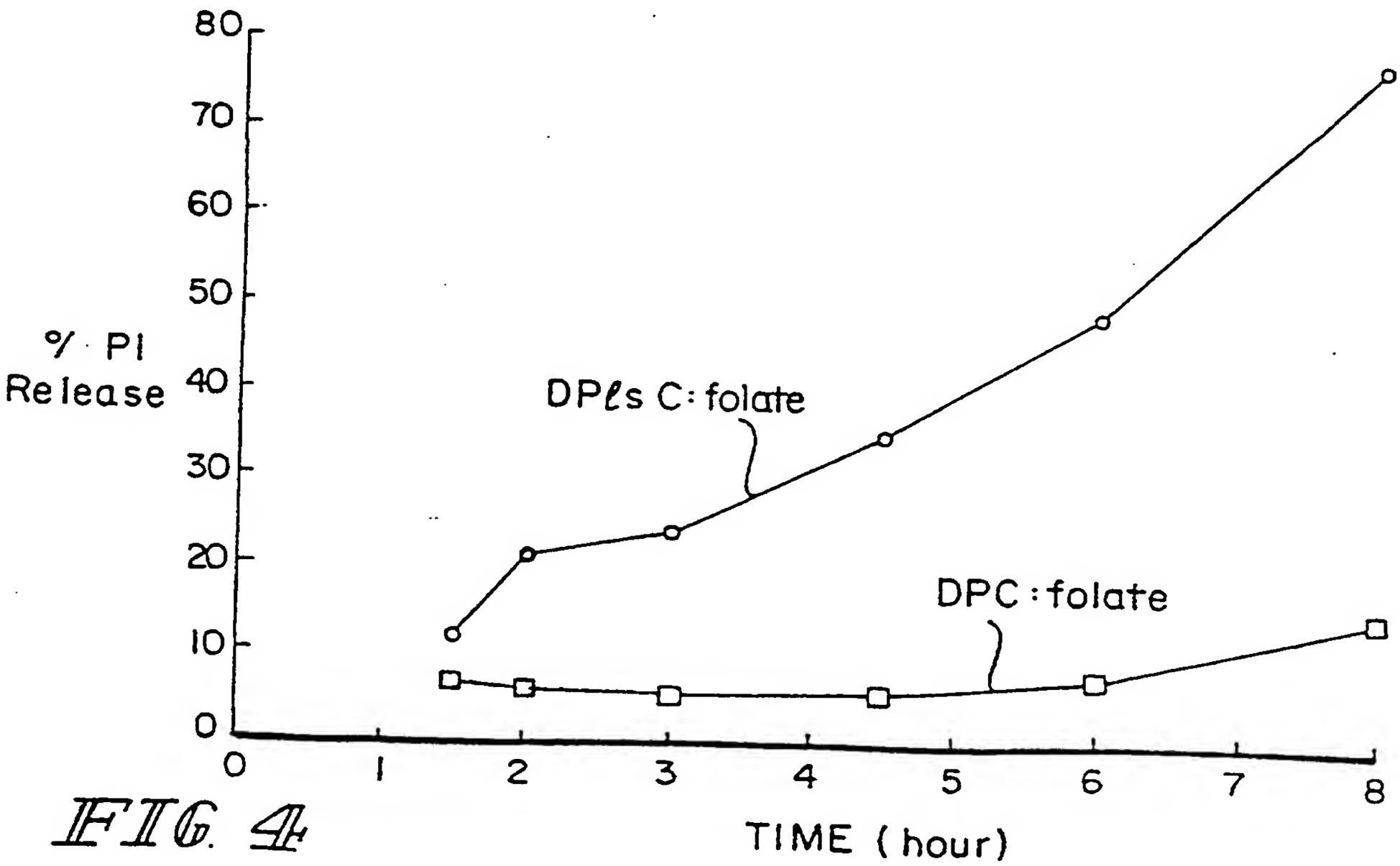

80
70
60
50
40
30
20
10
0
% PI Release
DPℓs C: folate
DPC: folate
0
1
2
3
4
5
6
7
8
TIME (hour)

FIG. 4

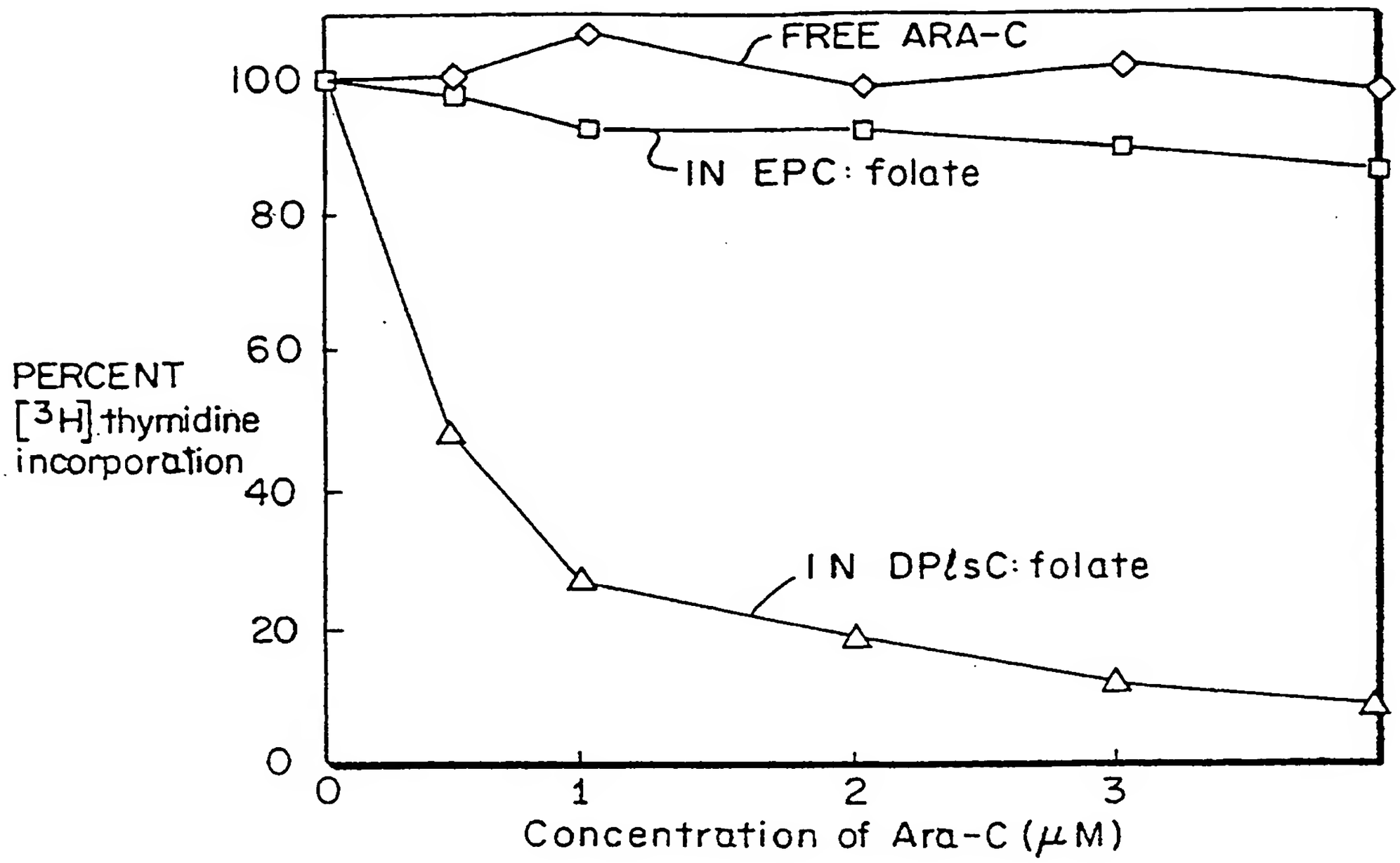

FREE ARA-C
IN EPC: folate
IN DPℓsC: folate
PERCENT
[3H] thymidine
incorporation
100
80
60
40
20
0
0
1
2
3
Concentration of Ara-C (μM)

FIG 5

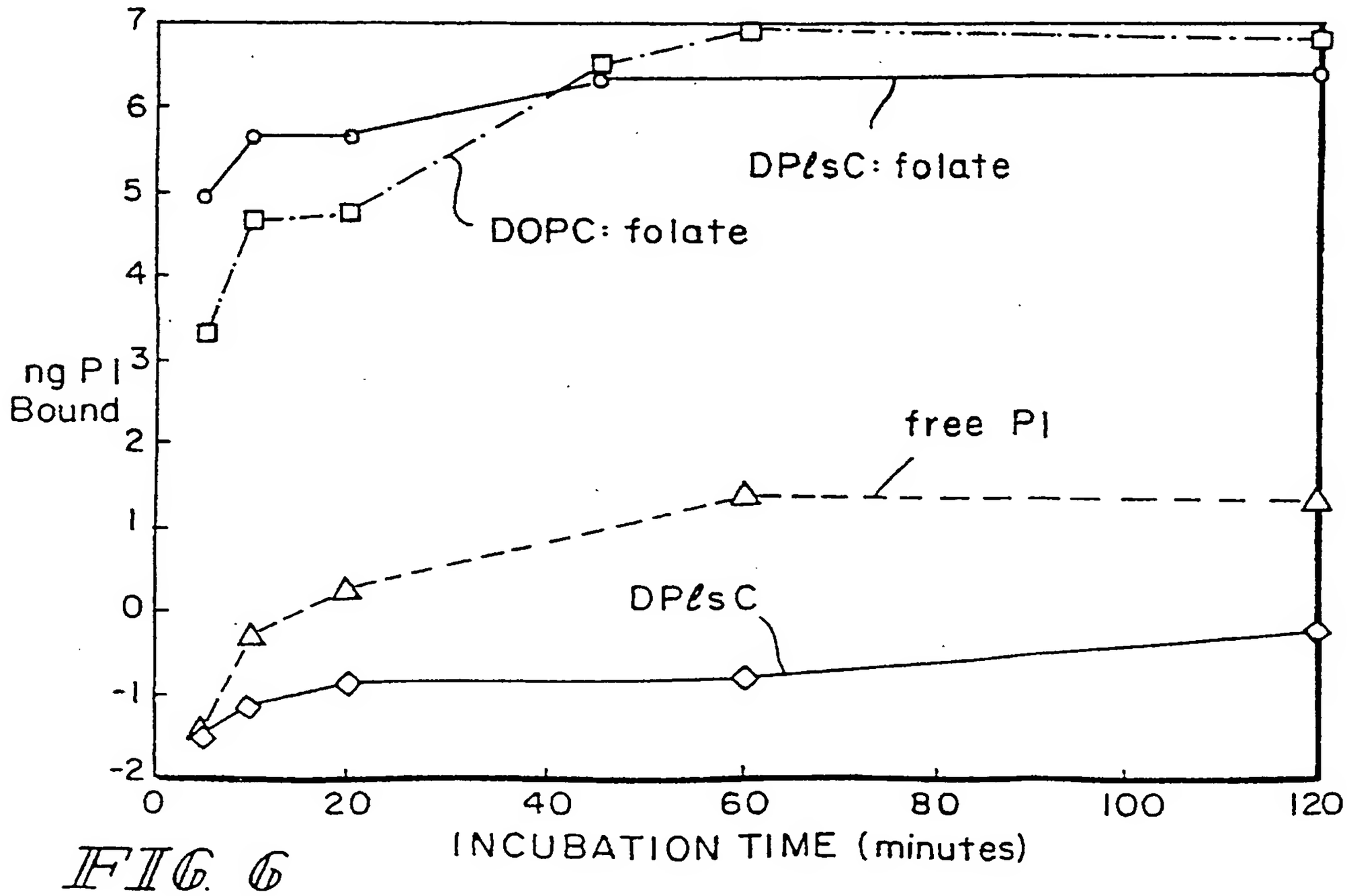

DPℓsC: folate
DOPC: folate
free PI
DPℓsC
ng PI
Bound
7
6
5
4
3
2
1
0
-1
-2
0
20
40
60
80
100
120
INCUBATION TIME (minutes)

FIG 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03077

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 9/127
US CL :424/450

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,2,77,913 A (THOMPSON et al.) 11 January 1994, abstract, columns 9-10, examples and claims.	1-14
Y	US 5,399,331 A (LOUGHREY et al.) 21 March 1995, abstract, examples and claims.	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 MAY 1997	24 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GOLLAMUDI S. KISHORE Telephone No. (703) 308-2351

Form PCT/ISA/210 (second sheet)(July 1992)★